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(54) Title: SIMPLIFIED MUTATION DETECTION			
(57) Abstract			
<p>The invention involves methods and materials for detecting a mutation in the genome of an organism. Specifically, the invention provides compositions of oligonucleotides and diagnostic kits that can be used to amplify nucleic acid sequences such that if heterologous amplified fragments are present, then stable nucleic acid branching structures are formed and detected from a cell lysate mixture containing the amplified nucleic acid. The formation of stable nucleic acid branching structures is particularly useful for providing a mechanism to detect heterologous alleles versus homozygous alleles since homozygous amplified products do not form stable nucleic acid branching structures. In addition, the invention provides methods of detecting heterologous Factor V alleles as well as homozygous wild-type and homozygous mutant Factor V alleles.</p>			

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## SIMPLIFIED MUTATION DETECTION

### BACKGROUND

#### 5 1. Technical Field

The invention relates to methods and materials involved in the rapid detection of mutations.

#### 2. Background Information

Many mutations in human genes exist and are associated with disease or 10 disease risk. For example, a single mutation in exon 10 of the cystic fibrosis gene, delta F508, is associated with cystic fibrosis. In addition, a point mutation in Factor V appears to be associated with thrombosis. Briefly, Factor V is a polypeptide involved in the coagulation cascade. Normally, activated protein C deactivates Factor V to regulate the cascade of coagulation events. One particular 15 mutant form of Factor V, known as Factor V Leiden, contains a single point mutation (G1691A) that renders the polypeptide resistant to activated protein C. Interestingly, Factor V Leiden is found in almost every ethnic group. For example, about 5-7% of the individuals of European or Scandinavian ancestry are carriers. In addition, about 1-2% of the population of other ancestries are carriers. Rather 20 strikingly, over 20% of patients with thrombosis are carriers. Thus, the mutation found in Factor V Leiden may be involved in blood disorders.

Current methods used to detect mutations such as Factor V Leiden (e.g., bidirectional PCR amplification of specific alleles (bi-PASA), or restriction 25 fragment length polymorphism (RFLP)), rely on allele specific primers to amplify DNA that is then analyzed by gel electrophoresis to detect the presence or absence of a specific fragment. The use of gel electrophoresis as a method of detection greatly increases the time required to analyze a sample and limits the number of

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samples that can be analyzed. Thus, any method that rapidly and accurately detects mutations and does not require gel electrophoresis would be desirable.

## SUMMARY

The invention involves methods and materials for detecting mutations.

- 5 Specifically, the invention is based on the discovery of a rapid and reliable detection assay that does not require nucleic acid purification or extraction. The elimination of the need to purify nucleic acid greatly reduces the time needed to perform an assay and does not compromise accuracy. In addition, this simplification of the detection assay reduces the number of steps required for
- 10 sample preparation and thus reduces the chances of operator error and contamination as well as exposure of the operator to potentially infectious material. Diagnostic kits containing the ingredients needed to perform this simplified mutation detection assay are provided by the invention. Such kits are particularly useful to diagnostic laboratory personnel since the kits can provide all the necessary
- 15 ingredients in a more uniform manner than otherwise would be available. For example, samples can be analyzed at separate facilities and the results accurately compared to each other since the ingredients in each kit can be prepared in a similar manner by a common vendor. This is particularly important for buffer solutions, oligonucleotide primers, and detection reagents. The invention also
- 20 provides specific oligonucleotide primers and nucleic acid amplification conditions that allow heterologous amplification products of the human Factor V gene to form stable branching structures. The formation of these stable branching structures is particularly useful for providing a mechanism to detect heterologous alleles versus homozygous alleles. In addition, the invention provides oligonucleotide primers as
- 25 well as assay conditions that allow for the rapid and accurate screening of multiple samples.

In general, the invention features a composition containing oligonucleotides. The composition amplifies a region of Factor V genomic sequence during a nucleic acid amplification reaction such that amplified products

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- that are heterologous form stable branching structures. In addition, the region contains a potential mutation site. The composition can contain two oligonucleotides that are identical in nucleic acid sequence (e.g., SEQ ID NO:1). Each of the two oligonucleotides can have a different label attached at the 5' end
- 5 (e.g., biotin and digoxigenin). Further, the composition can contain two oligonucleotides each having a 5' portion and a 3' portion. The 5' portions of the two oligonucleotides are different in nucleic acid sequence, and the 3' portions of the two oligonucleotides are identical in nucleic acid sequence. The 3' portions hybridize to a sequence flanking the mutation site during the nucleic acid
- 10 amplification reaction. One of the two oligonucleotides can have a nucleic acid sequence as set forth in SEQ ID NO:2, 3, 4, 5, 6, or 7. The amplified products can be less than about 300 bases in length in which case one of the oligonucleotides can have a nucleic acid sequence as set forth in SEQ ID NO:4, 5, 6, or 7. Alternatively, the
- 15 oligonucleotides of the composition can produce false positive results in a LOCI mutation detection assay less frequently than when Dig-Primer1, Bt-Primer1, Primer2, and Primer3 primers are used in the LOCI mutation detection assay. In this case, the oligonucleotides can include Dig-Primer1, Bt-Primer1, Primer4, and Primer5.
- 20 Another aspect of the invention features an oligonucleotide that consists of the sequence as set forth in SEQ ID NO:1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14.

In another aspect, the invention features a method of detecting heterologous Factor V alleles in an organism. The method includes amplifying a

25 region of Factor V nucleic acid from the organism using oligonucleotides such that amplified products that are heterologous form stable branching structures, and detecting the presence or absence of the stable branching structures. The region contains a potential mutation site and the presence of the stable branching structures is indicative of the presence of the heterologous Factor V alleles in the

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organism. LOCI can be used for the detecting step. Alternatively, a sandwich immunoassay can be used for the detecting step.

- Another embodiment of the invention features a method of detecting homozygous mutant Factor V alleles in an organism. The method includes
- 5 amplifying a region of Factor V nucleic acid from the organism using oligonucleotides to form amplification products; contacting the amplification products with a solution to form a mixture; and detecting the presence or absence of stable branching structures in the mixture. The region contains a potential mutation site and the solution contains homozygous wild-type Factor V products
- 10 amplified using the oligonucleotides. The stable branching structures are formed between the amplification products and the homozygous wild-type Factor V products. In addition, the presence of the stable branching structures is indicative of the presence of the homozygous mutant Factor V alleles in the organism. LOCI can be used for the detecting step. Alternatively, a sandwich immunoassay can be
- 15 used for the detecting step.

- Another aspect of the invention features a diagnostic kit for nucleic acid extraction-free detection of heterologous alleles in an organism (e.g., human). The kit contains a cell lysis reagent and two oligonucleotides. The two oligonucleotides are identical in nucleic acid sequence (e.g., SEQ ID NO:1) with each of the two
- 20 oligonucleotides having a different label attached at the 5' end (e.g., biotin and digoxigenin). In addition, the two oligonucleotides hybridize to a first region of genomic sequence during a nucleic acid amplification reaction and the first region flanks a potential mutation site. The cell lysis reagent can contain an ingredient that inactivates a polymerase inhibitor. The genomic sequence can contain a Factor
- 25 V genomic sequence. Alternatively, the genomic sequence can contain a cystic fibrosis genomic sequence. Further, the kit can contain two additional oligonucleotides each having a 3' portion and a 5' portion. The 5' portions are different in nucleic acid sequence, and the 3' portions are identical in nucleic acid sequence. The 3' portions hybridize to a second region of the genomic sequence
- 30 during the nucleic acid amplification reaction, and the second region is located on a

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side of the potential mutation site that is opposite the side of the first region. The second region can be downstream or upstream of the potential mutation site. One of the two additional oligonucleotides can have a sequence as set forth in SEQ ID NO:2, 3, 4, 5, 6, or 7.

- 5 Another aspect of the invention features a method of comparing nucleic acid sequences of alleles in an organism. The method includes amplifying a nucleic acid region of the alleles in a cell lysate to form a cell lysate mixture having amplification products; analyzing the cell lysate mixture for the presence or absence of stable nucleic acid branching structures; and classifying the alleles as
- 10 heterologous if the stable nucleic acid branching structures are present in the cell lysate mixture.

- In another embodiment, the invention features a method of detecting homozygous mutant alleles in an organism. The method includes amplifying a nucleic acid region of the alleles in a cell lysate using oligonucleotides to form a
- 15 cell lysate mixture having amplification products; contacting the cell lysate mixture with a solution to form a second mixture; analyzing the second mixture for the presence or absence of stable nucleic acid branching structures; and classifying the alleles as homozygous mutant alleles if the stable nucleic acid branching structures are present in the second mixture. The solution contains homozygous wild-type
- 20 products amplified from homozygous wild-type alleles using the oligonucleotides.

- Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the
- 25 present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

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Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

#### DESCRIPTION OF DRAWINGS

Figure 1 is a diagram depicting the nucleic acid branching structure that  
5 is stabilized when formed from heterologous alleles.

Figure 2 is a diagram depicting the nucleic acid branching structure formed from heterologous alleles using primers that are tagged with either biotin (Bt) or digoxigenin (Dig).

Figure 3 is a diagram depicting a process of detecting stabilized  
10 branching structures.

#### DETAILED DESCRIPTION

The invention provides methods and materials related to the detection of a mutation in a genome of an organism. Specifically, the invention provides a simplified method for detecting mutations as well as diagnostic kits containing the  
15 ingredients needed to perform a simplified mutation detection assay. In addition, the invention provides oligonucleotide primers as well as assay conditions that allow for rapid and accurate detection of Factor V Leiden and a mutation in the cystic fibrosis gene.

In general, the simplified mutation detection method provided by the  
20 invention involves the detection of heterologous alleles (i.e., alleles differing in sequence) from a nucleic acid amplification reaction mixture prepared directly from a cellular sample (e.g., a cell lysate). Thus, the method does not involve the purification of nucleic acid. The term "cell lysate" as used herein refers to any solution that contains the cellular constituents (e.g., proteins, lipids, carbohydrates,  
25 and nucleic acid) produced upon disruption of a cell. For example, nucleic acid isolated from a solution of disrupted cells is not a cell lysate. Cell lysate solutions, however, can include, without limitation, a solution of lysed cells that is treated

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such that selected molecules are removed or rendered inactive. It follows that this solution remains substantially "crude" with respect to most purified cellular constituents. For example, a cell lysate can be a solution of lysed cells that is treated with an agent that inactivates or removes polymerase inhibitors (U.S. Patent 5 No. 5,284,940). In addition, a cell lysate can be a solution of lysed cells that is treated with an anti-coagulant (U.S. Patent No. 5,501,963).

Any method can be used to lyse cells in a cellular sample. For example, osmotic shock, sonication, heating, physical disruption, microwave treatment, and alkaline lysis are methods that can be used to lyse cells. In addition, the 10 GeneReleaser™ kit (BioVentures, Inc) can be used to produce a cell lysate. A cellular sample can be any type of cellular sample including, without limitation, blood samples, urine sedimentary cell samples, throat swabs, and skin samples. For example, a cellular sample such as whole blood can be collected from a human, lysed, and the nucleic acid region of interest amplified by a nucleic acid 15 amplification reaction. A nucleic acid amplification reaction can be any reaction provided the desired nucleic acid region is amplified. For example, a PCR reaction can be performed using Taq polymerase.

In general, PCR refers to a procedure or technique in which target nucleic acid is amplified in a manner similar to that described in U.S. Patent No. 20 4,683,195, and subsequent modifications of the procedure described therein. Generally, sequence information from the ends of the region of interest or beyond are used to design oligonucleotide primers that are identical or similar in sequence to opposite strands of a potential template to be amplified. Using PCR, a region of nucleic acid can be amplified from RNA or DNA. For example, a region of 25 nucleic acid can be isolated by PCR amplification from total cellular RNA, total genomic DNA, and cDNA as well as from bacteriophage sequences, plasmid sequences, viral sequences, and the like. When using RNA as a source of template, reverse transcriptase can be used to synthesize complimentary DNA strands.

The term "nucleic acid" as used herein encompasses both RNA and 30 DNA, including cDNA, genomic DNA, and synthetic (e.g., chemically synthesized)

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DNA. The nucleic acid can be double-stranded or single-stranded. Where single-stranded, the nucleic acid can be the sense strand or the antisense strand. In addition, nucleic acid can be circular or linear.

- The products resulting from a nucleic acid amplification reaction are
- 5 collectively referred to as amplification products or amplified products. Such products can be subjected to conditions that promote the formation of stable branching structures. For example, amplification products can be incubated at about 96°C for about 2 minutes to melt any double stranded nucleic acid, and then can be incubated at about 65°C for about 30 minutes to initiate stable branching
- 10 structure formation after which the products can be maintained at 4°C until analyzed. For the purpose of this invention, a stable branching structure refers to a Holliday Junction or cruciform structure of nucleic acid whose dissociation is blocked by at least one mismatch within the amplified region of nucleic acid (Figure 1). Thus, the amplification of a region of nucleic acid from homologous
- 15 alleles would not result in stable branching structures since the amplified region of nucleic acid does not contain a mismatch that can prevent dissociation. On the other hand, the amplification of a region of nucleic acid from heterologous alleles would result in stable branching structures since the amplified region of nucleic acid contains a mismatch that can prevent dissociation.
- 20 In general, a nucleic acid amplification reaction that is used to generate stable branching structures contains four oligonucleotide primers. Two of the four oligonucleotide primers hybridize, during the nucleic acid amplification reaction, to a genomic sequence upstream of a potential mutation site. These two primers have identical sequences but different 5' labels. Examples of 5' labels include, without
- 25 limitation, binding agents such as antigens and ligands. For example, an antigen such as digoxigenin and a ligand such as biotin can be used as 5' labels (Figure 2).
- The other two oligonucleotide primers have identical 3' portions and different 5' portions. The identical 3' portions of each primer can hybridize, during the nucleic acid amplification reaction, to any genomic sequence
- 30 downstream of a potential mutation site. The different 5' portions of each primer

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can have a sequence such that this portion does not hybridize to any of the genomic sequences flanking the potential mutation site. Thus, the two upstream primers can have different 5' labels and the two downstream primers can have different 5' sequences. It is noted that these primers can be switched such that the 5 two upstream primers have different 5' sequences and the two downstream primers have different 5' labels.

- A potential mutation site is any nucleic acid position that is found or suspected to have a particular base in one organism that is different from the particular base found at that same position in another organism of the same species.
- 10 Thus, any polymorphism is considered a potential mutation site. As described herein, any region of the human genome can be a region of nucleic acid containing a potential mutation site. For example, the region can be from genes associated with disease such as  $\beta$ -APP, ApoE4, cystic fibrosis, Factor V, and HFE (hemochromotosis) genes as well as oncogenes such as the RET proto-oncogene.
- 15 Other examples of genes that can be analyzed are listed in Tables I and II.

Table I. Gene associated with venous thrombosis and cardiovascular disease

<b>Venous Thrombosis</b>		
<u>Gene</u>	<u>#Mutations</u>	<u>Prevalence (VT)</u>
Factor V Leiden	2 (1 rare)	45% (European)
20 Prothrombin Elevated	1	18% (European)
Dysfibrinoginemia	>11	1%
Protein S Deficiency	>69	5.7%
Antithrombin Deficiency	>79	4.3%
Protein C Deficiency	>160	5.7%
25 Unknown	?????	~20%

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**Cardiovascular Disease**

<u>Gene</u>	<u>#Mutations</u>
apoA	21
apoA-4	4
5 apoB	17
apoC	11
apoE	29
LP(a)	2
10 Hyperlipoproteinemia (Lipoprotein Lipase)	35
Hypercholesterolemia (LDL Receptor)	54

Table II. Addition gene associated with disease

<u>Condition</u>	<u>Gene</u>	<u>Mutations</u>	<u>Prevalence</u>	<u>Comments</u>
Hemochromatosis	HLA-H	1	1/14 Heterozygous 1/250 Homozygous	Therapy available
5 Hyperhomo-cysteinuria	MTHFR	7		Therapy available
Hyperhomo-cysteinemia	MTHFR	1	9-17% Homozygous 31-39% Heterozygous	Therapy available
Hyperhomo-cysteinemia	Cystathione- $\beta$ -synthase	3		Therapy available
10 Medium Chain Fatty Acid Coenzyme A Reductase		>9 1 ~90%		Therapy available Possible new-born screening
Pancreatic Cancer	k-RAS	1	~20,000 new cases annually	Screen: new diabetics, pancreatitis, select other cancer syndromes
15 Multiple Endocrine Neoplasia Type II/Familial Medullary Thyroid Cancer	RET proto-oncogene	28		
20 Cancer	p53	numerous	25-50% of cancers	

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Several different methods can be used to detect the presence or absence of stable branching structures when nucleic acid is amplified from purified DNA or a cell lysate. Again, the presence of stable branching structures indicates that the amplified region was generated from heterologous alleles that were used as 5 templates during the amplification reaction. In general, the invention provides detection methods that involve fluid phase detection assays and not gel electrophoresis. For example, luminescence oxygen channeling immunoassays (LOCI) can be used to detect the presence or absence of stable nucleic acid branching structures from a cell lysate mixture containing amplified nucleic acid

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(Figure 3). In addition, sandwich immunoassays can be used to detect the presence or absence of stable nucleic acid branching structures.

It is noted that the presence of heterologous alleles can be detected from the cell lysate mixtures described herein. For example, denaturing reverse-phase  
5 high-performance liquid chromatography (D-HPLC) can be used to discriminate between homo- and hetero- duplex nucleic acid within such mixtures, while MutS-based assays can be used to detect heteroduplex nucleic acid within such mixtures. Briefly, MutS-based assays use a bacterial polypeptide (MutS) that specifically binds the mis-match site of heteroduplex nucleic acid (Parsons and Heflich, *Mutat*  
10 *Res* 374(2):277-85 (1997) and Ellis *et al.*, *Nucleic Acids Res* 22(13):2710-11 (1994)).

Using a stable nucleic acid branching structure assay, a Factor V Leiden analysis can proceed as follows. A fragment containing the point of mutation in Factor V is specifically amplified using PCR. Under certain conditions, the  
15 amplified DNA of a heterozygous carrier (i.e., an individual having heterologous Factor V alleles) forms a stable nucleic acid branching structure that brings a donor/acceptor together such that a chemiluminescence signal is generated (Figures 1-3). A positive chemiluminescence signal is observed first in a heterozygous specimen. Amplified DNA from an individual having homozygous wild-type  
20 Factor V, or homozygous Factor V Leiden alleles, results in a negative signal. If fragments from an individual homozygous for Factor V Leiden, however, are mixed with fragments from a known wild-type Factor V sample, then an artificial heterozygous DNA sample will be generated from an original homozygous specimen. Therefore, a homozygous Factor V Leiden carrier is detected by the  
25 mixing test incorporated as part of this assay. The presence of stable branching structures can be detected using a LOCI format or a microtitre plate format. In both cases, streptavidin and anti-digoxigenin can be used to identify the presence of stable branching structures containing both biotin and digoxigenin.

A region of genomic Factor V nucleic acid containing two potential  
30 mutation sites is as follows:

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- ttatttattatcatgaaataacttgcaaatgaaaacaatttgaatatatttcgtcaGGCAGGAACAAACACCAT  
GATCAGAGCAGTTCAACCAGGGAAACCTATACTTATAAGTGGAACATCT  
TAGAGTTGATGAACCCACAGAAAATGATGCCAGTGCTTAACAAGACCA  
TAATCAGTGACGTGGACATCATGARAGACATCGCCTCTGGGCTAATAGG  
5 ACTACTTCTAATCTGTAAGAGCAGATCCCTGGACAGGC~~R~~AGGAATACAGgt  
attttgccttgaagtaacccttcagaaattctgagaatttctggctagaacatgttaggtctcctggctaaataatggggcat  
ttccctcaagagaacagtaattgtcaagtagtccttttagcaccagtgtgataacatttattcttttttttgtct (SEQ ID  
NO:15). The first stretch of lower case bases represents sequences from intron 9,  
while the second stretch of lower case bases represents sequence from intron 10.  
10 The capitalized bases are sequences from exon 10 with the first bold R being  
position 1628 and the second bold R being position 1691 (the Factor V Leiden  
position). An R indicates either a G or A, while the wild-type Factor V would  
have a G at both of these positions.

The invention will be further described in the following examples, which  
15 do not limit the scope of the invention described in the claims.

## EXAMPLES

### Example 1 - Factor V Leiden Detection from Purified DNA

This protocol combined with the selection of the primers selectively amplifies the region of the Factor V Leiden mutation, and eliminates non-specific binding as well as the amplification of genes having homology to Factor V, such as Factor VIII. In addition, the stable nucleic acid branching structures formed between heterozygous strands of DNA generated by this protocol were detected by a LOCI method without having to run electrophoresis gels.

Four primers were designed. The two upper primers target the 5'-end of  
25 the exon 10 of the Factor V gene. Each of these two primers was designed to have the same sequence (5'-CAGGCAGGAACAAACACCATGAT-3'; SEQ ID NO:1) with a different binding agent attached at the 5'-end: one having digoxigenin (Dig-Primer1) and the other having biotin (Bt-Primer1). Both Dig-Primer1 and Bt-Primer1 were obtained from OLIGOSETC (Wilsonville, OR) in a purified and

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lyophilized form. Before use, each primer was dissolved in distilled water to form a stock solution having a concentration of 25  $\mu$ M. The stock solutions of these primers were stored at -20°C.

The two lower primers (Primer2 and Primer3) also target exon 10 of the Factor V gene. These two primers were designed to have an identical sequence at the 3'-end and a different sequence at the 5'-end of the primer. The sequence of Primer2 (SEQ ID NO:2) and Primer3 (SEQ ID NO:3) is as follows with the identical sequences being underlined:

5'-ACCATGCTCGAGATTACGAGTTATCACACTGGTGCTAAAAAGGA-3'  
10 (SEQ ID NO:2) 5'-  
GATCCTAGGCCTACGTATTTATCACACTGGTGCTAAAAAGGA-3' (SEQ  
ID NO:3)

Both lower primers (Primer2 and Primer3) were synthesized by the CORE Facility at Mayo Foundation. Briefly, the primers were desalted using a 15 Pharmacia NAP-25 column and eluted from the column with distilled water in 1 mL fractions. The absorption at 260 nm ( $A_{260}$ ) of each fraction was measured using an HP8452A Diode Array Spectrophotometer and a quartz micro cell (HP QS 0.200) that has a light path of 0.200 cm. Fractions with significant  $A_{260}$  of each primer were pooled and concentrated using a Speed-Vac. The  $A_{260}$  was measured 20 and the final concentration C ( $\mu$ g/mL) of each primer was calculated using the formula:  $C = 5 \times 33 \times A_{260} \mu\text{g/mL}$ . The molar concentration ( $C_M$ ) can be calculated using  $C_M = C \times 1000 / (325 \times N) \mu\text{M}$ , where N is the number of bases in the primer. Stock solutions of these primers were stored at -20°C.

In general, the following methods involved four steps: (1) purification of 25 nucleic acid from a cellular sample, (2) amplification of target DNA by PCR, (3) formation of branched DNA between strands of heterozygous alleles, and (4) detection of the branched DNA using the luminescence oxygen channeling immunoassay (LOCI). These steps were used to detect a heterozygous carrier while a mixing test was done to detect a homozygous carrier.

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Standard nucleic acid isolation techniques (AGTC Kit®; Analytic Genetics Testing Center) were used to isolate genomic DNA from white blood cells. For each patient, genomic DNA was precipitated by ethyl alcohol and re-dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The concentration of 5 genomic DNA from each patient varied, but was generally around 50 mg/ $\mu$ L. It was not necessary to adjust each genomic DNA to an exact concentration as long as it was around 50 ng/ $\mu$ L.

Once the genomic DNA was isolated, the target DNA was amplified using a GeneAmp PCR System 2400 (Perkin Elmer) PCR machine. To prevent 10 PCR carry-over and contamination, the PCR reagents and DNA templates (especially DNA amplicon) were physically separated in two different rooms. The PCR reagents included nuclease-free water (PT#P119C; Promega), PCR tubes (MicroAmp Reaction Tubes; Perkin Elmer), 10X polymerase buffer, 10mM dNTP mix (BRL), 50 mM MgCl<sub>2</sub>, a set of micropipets, and pipette tips. The 10X 15 polymerase buffer was supplied by Stratagene with the native *Pfu* DNA polymerase and contained 200 mM Tris-HCl (pH 8.0); 20 mM MgCl<sub>2</sub>; 100 mM KCl; 60 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 1% Triton X-100; and 100  $\mu$ g/ml nuclease-free BSA. The 50mM MgCl<sub>2</sub> was either obtained from BRL or made in large quantities to maintain a consistent concentration. In addition, the 10X polymerase buffer, 10mM dNTP 20 mix (BRL), and 50 mM MgCl<sub>2</sub> were stored in at -20°C.

Two different forms of the proofreading DNA polymerase *Pyrococcus furiosus* (*Pfu* polymerase) were used: native and cloned. Both were obtained from Stratagene and had a concentration of 2.5 U/ $\mu$ L. The native *Pfu* polymerase can give the best signal-to-noise ratio, although constant supply can be a problem. In 25 addition, two different protocols were developed, one for use with the native *Pfu* polymerase and another for use with the cloned *Pfu* polymerase.

The following methods and materials were used in conjunction with the native *Pfu* polymerase. Fifteen minutes before use, the 10X polymerase buffer, 10 mM dNTP mix, 50 mM MgCl<sub>2</sub>, and a primer mix was warmed to room 30 temperature. The primer mix was made as follows. A 100  $\mu$ L solution of each

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primer (Dig-Primer1; Bt-Primer1; Primer2; and Primer3) was made in double distilled water to have a concentration of 12.5  $\mu$ M. An equal volume of each 100  $\mu$ L solution then was mixed together to make the primer mix. The final concentration of each primer in this primer mix was 3.125  $\mu$ M.

- 5        The PCR reaction buffer (L-Buffer), polymerase working solution (U-Buffer), and DNA dilution buffer (G-Buffer) were made in separated 250- $\mu$ L Eppendorf tubes. For 10 PCR reactions each having a total volume of 50  $\mu$ L, the L-Buffer, U-Buffer, and G-Buffer was made as follows:

	<b>L-Buffer</b>	<b>U-Buffer</b>	<b>G-Buffer</b>
<b>Nuclease-free water</b>	61.25 $\mu$ L	103.75 $\mu$ L	207.5 $\mu$ L
<b>10X polymerase buffer</b>	12.5 $\mu$ L	12.5 $\mu$ L	25 $\mu$ L
<b>50 mM MgCl<sub>2</sub></b>	8.75 $\mu$ L	8.75 $\mu$ L	17.5 $\mu$ L
<b>Primer mix</b>	40 $\mu$ L		
<b>10 mM dNTP mix</b>	5 $\mu$ L		
<b>Native Pfu polymerase</b>		6 $\mu$ L	
<b>Total volume</b>	127.5 $\mu$ L	131 $\mu$ L	250 $\mu$ L

Once the ingredients were added, the tubes were mixed gently by mid-speed vortex.

- To setup each PCR reaction, 12.5  $\mu$ L of L-Buffer was transferred to each labeled PCR tube and 25  $\mu$ L of G-Buffer was transferred to each labeled DNA tube (250- $\mu$ L Eppendorf tube). The U-Buffer was maintained on ice and the labeled PCR and DNA tubes as well as the U-Buffer were brought to the PCR room where 1  $\mu$ L of genomic DNA was added to each labeled DNA tube. The labeled DNA tubes then were vortexed and the contents transferred to each labeled PCR tube. One positive control (heterozygous) and one negative control (wild type) DNA specimen was included in each PCR run. To start the reactions, 12.5  $\mu$ L of U-buffer was added to each labeled PCR tube and the tubes were mixed gently by mid-speed vortex before being placed on the PCR machine.

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During the PCR thermal cycling procedure, the PCR tubes were subjected to a starting temperature of 95°C for 4 minutes, followed by 5 cycles (95°C for 30 seconds, 68°C for 45 seconds, and 72°C for 1 minute), followed by 25 cycles (95°C for 30 seconds, 70°C for 45 seconds, and 72°C for 1 minute), 5 followed by 7 minutes at 72°C. To melt the DNA double strands, each PCR tube was incubated at 96°C for 2 minutes. In addition, the branched DNA formation was initiated by placing the denatured DNA at 65°C for 30 minutes and then incubating the reaction at 4°C for 30 minutes.

The following methods and materials were used in conjunction with the 10 cloned *Pfu* polymerase. Fifteen minutes before use, the 10X polymerase buffer, 10 mM dNTP mix, 50 mM MgCl<sub>2</sub>, and a primer mix was warmed to room temperature. The primer mix was made as follows. A 100 µL solution of each primer (Dig-Primer1; Bt-Primer1; Primer2; and Primer3) was made in double distilled water to have a concentration of 12.5 µM. An equal volume of each 100 15 µL solution then was mixed together to make the primer mix. The final concentration of each primer in this primer mix was 3.125 µM.

The PCR reaction buffer (L-Buffer) and polymerase working solution (U-Buffer) were made in separated 250-µL Eppendorf tubes. For 10 PCR reactions each having a total volume of 50 µL, the L-Buffer and U-Buffer was made as 20 follows:

	L-Buffer	U-Buffer
Nuclease-free water	305 µL	112.5 µL
10X polymerase buffer	37.5 µL	12.5 µL
50 mM MgCl <sub>2</sub>	36 µL	
Primer mix	40 µL	
10 mM dNTP mix	5 µL	
Cloned <i>Pfu</i> polymerase		6.5 µL
Total volume	423.5 µL	131.5 µL

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Once the ingredients were added, the tubes were mixed gently by mid-speed vortex.

To setup each PCR reaction, 37.5  $\mu$ L of L-Buffer was transferred to each labeled PCR tube and the U-Buffer was maintained on ice. The labeled PCR 5 and U-Buffer then were brought to the PCR room where 1  $\mu$ L of genomic DNA (around 50 ng/ $\mu$ L) was added to each labeled PCR tube. The labeled PCR tubes then were mixed by mid-speed. Again, one positive control (heterozygous) and one negative control (wild type) DNA specimen was included in each PCR run. To start the reactions, 12.5  $\mu$ L of U-buffer was added to each labeled PCR tube 10 and the tubes were mixed gently by mid-speed vortex before being placed on the PCR machine.

During the PCR thermal cycling procedure, the PCR tubes were subjected to a starting temperature of 95°C for 4 minutes, followed by 5 cycles (95°C for 30 seconds, 69°C for 45 seconds, and 72°C for 1 minute), followed by 15 25 cycles (95°C for 30 seconds, 70°C for 45 seconds, and 72°C for 1 minute), followed by 7 minutes at 72°C. To melt the DNA double strands, each PCR tube was incubated at 96°C for 2 minutes. In addition, the branched DNA formation was initiated by placing the denatured DNA at 65°C for 30 minutes and then incubating the reaction at 4°C for 30 minutes.

20 The PCR primers Dig-Primer1, Bt-Primer1, Primer2, and Primer3 as well as the two PCR protocols were designed to amplify a DNA fragment of about 400 base pairs that contained the region surrounding position 1691 of exon 10 of the Factor V gene. Analysis of the PCR products revealed that the primers and protocols amplified a 400 base pair fragment. Briefly, 2% agarose gel 25 electrophoresis of the PCR products generated by either the native or the cloned *Pfu* protocol resolved a single band of DNA having 400 base pairs, confirming that each PCR protocol specifically amplifies the targeted region of exon 10 of the Factor V gene.

To detect heterozygous carriers, the PCR products were examined using 30 a luminescence oxygen channeling immunoassay (LOCI) method as follows.

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Briefly, the LOCI reagents were 1X LOCI buffer (10 mM Tri-HCl, pH 8.3; 4 mM MgCl<sub>2</sub>; 50 mM KCl; and 200 µg/ml nuclease-free BSA) and two types of detection beads (Behring Diagnostics). The blue detection beads were sensitizer beads coated with streptavidin (2300 streptavidin/bead at a concentration of 500 µg/ml in 1X  
5 LOCI buffer). The yellow beads were acceptor or chemiluminescent beads coated with anti-digoxigenin antibody (2.5 mg/ml in 1X LOCI buffer). Each of these LOCI reagents were stored in a black box at 4°C. To perform the LOCI reaction, 4 µL of the PCR amplification product from each PCR tube were transferred to each LOCI detection tube (a labeled 650-µL Eppendorf tube). An empty tube was  
10 included in each assay as an assay blank. The LOCI detection tubes then were brought to a dark room and a detection solution (1 mL of 1 x LOCI buffer; 5 µL of the blue beads; and 5 µL of the yellow beads) prepared and mixed by mid-speed vortex. Once prepared, 100 µL of the detection solution were transferred to each LOCI detection tube and the tubes mixed gently by low speed vortex. After  
15 mixing, the LOCI detection tubes were incubated at 37°C for 30 minutes. After incubation, the LOCI signal was read using a LOCI machine (MPL-250; Behring). A high LOCI reading indicates that the specimen is heterozygous for Factor V Leiden, while a low LOCI reading indicates that the specimen is either homozygous for Factor V Leiden or wild-type.  
20 Since low LOCI readings mean the sample is either homozygous for Factor V Leiden or wild-type, a mixing test was performed to detect homozygous carriers. Briefly, 10 µL of each PCR product giving a low LOCI reading was mixed with 10 µL of the PCR product from a known negative control. The known negative control contained DNA that was homozygous for wild-type Factor V. To  
25 melt all the double stranded DNA, the mixed DNA samples were heated at 96°C for 2 minutes. After melting, the mixed DNA samples were incubated at 65°C for 30 minutes to form branched DNA, followed by chilling at 4°C for 30 minutes. After chilling the samples for 30 minutes, LOCI readings were taken. At this point, a high LOCI reading indicates that the specimen is homozygous for Factor V

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Leiden, while a low LOCI reading indicates that the specimen is wild-type for Factor V.

A signal to noise ratio (S/N) was determined to minimize variations between assays. Specifically, the S/N was obtained by dividing the LOCI reading obtained from each PCR product by the LOCI reading obtained from the assay blank. The S/N was above 10 for heterozygous carriers and below 5 for wild-type DNA when either the native or cloned *Pfu* Protocol was used.

Twenty (20) known heterozygous DNA specimen were tested to determine the sensitivity of detecting heterozygous DNA. For all 20 samples, high S/N results were obtained, indicating that the sensitivity for a true heterozygous carrier was 100%. In addition, 11 known wild-type specimen were tested to determine specificity. For all 11 samples, low S/N results were obtained, indicating that the specificity for a true wild-type specimen was 100%. Further, seven known homozygous DNA samples were tested by the direct assay as well as by the mixing test to determine the sensitivity of detecting homozygous DNA. For all seven sample, low S/N results were obtained for the direct assay and high S/N results obtained upon mixture with a known wild-type DNA sample, indicating that the sensitivity for a true homozygous carrier was 100%. Performing the mixing test on a known wild-type specimen resulted in a low S/N result.

Fourteen (14) specimen were tested using the native *Pfu* protocol in a blind fashion. The S/N results obtained then were compared with results obtained using bi-PASA, a standard assay performed in coagulation labs. Five heterozygous and nine wild-type specimen were correctly identified.

In addition, 50 specimen were tested using the cloned *Pfu* protocol in a blind fashion. The S/N results obtained then were compared with those from bi-PASA. Twenty-four (24) heterozygous and 26 wild-type specimen were correctly identified. It is noted that one disagreement between the LOCI method and bi-PASA method was observed. Re-evaluation using both methods, however, revealed that the original result obtained by the bi-PASA method was incorrect. Thus, the

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second analysis confirmed that the original result obtained using the LOCI methods described herein was correct.

In a separate analysis, a total of 137 samples were analyzed in blind fashion. Nine samples produced positive results but were negative by the bi-PASA 5 technique (false positives). To eliminate the false positive results, additional oligonucleotide primers were designed. These primers were designed to amplify a 260 base pair fragment of nucleic acid spanning the G1691 site of Factor V, eliminating much of the intronic sequence amplified by the Dig-Primer1, Bt- Primer1, Primer2, and Primer3 primers.

10 In this analysis, the two upper primers were again Dig-Primer1 and Bt- Primer1. The two lower primers (Primer4 and Primer5), however, were different from Primer2 and Primer3. Specifically, Primer4 and Primer5 were designed to have an identical sequence at the 3'-end and a different sequence at the 5'- end of the primer. The sequence of Primer4 (SEQ ID NO:4) and Primer5 (SEQ ID 15 NO:5) is as follows with the identical sequences being underlined:

5'-ACCATGCTCGAGATTACGAGGGGTACTTCAAGGACAAATA-3' (SEQ ID NO:4)

5'-GATCCTAGGCCTCACGTATTGGTTACTTCAAGGACAAATA-3' (SEQ ID NO:5).

20 The PCR reactions and LOCI detection methods were as described above with the following changes. The PCR reaction buffer (L-Buffer) and polymerase working solution (U-Buffer) were made in separated 250- $\mu$ L Eppendorf tubes. For 10 PCR reactions each having a total volume of 50  $\mu$ L, the L-Buffer and U-Buffer was made as follows:

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	L-Buffer	U-Buffer
<b>Nuclease-free water</b>	316.8 $\mu$ L	130 $\mu$ L
<b>10X polymerase buffer</b>	40 $\mu$ L	7.9 $\mu$ L
<b>50 mM MgCl<sub>2</sub></b>	30 $\mu$ L	
<b>Primer mix</b>	16.8 $\mu$ L	
<b>10 mM dNTP mix</b>	4.18 $\mu$ L	
<b>Native <i>Pfu</i> polymerase</b>		6.25 $\mu$ L
<b>Total volume</b>	407.8 $\mu$ L	144.15 $\mu$ L

5

To setup each PCR reaction, 37.5  $\mu$ L of L-Buffer was transferred to each labeled PCR tube and the U-Buffer was maintained on ice. The labeled PCR 10 and U-Buffer then were brought to the PCR room where 1  $\mu$ L of genomic DNA (around 50 ng/ $\mu$ L) was added to each labeled PCR tube. The labeled PCR tubes then were mixed by mid-speed. Again, one positive control (heterozygous) and one negative control (wild type) DNA specimen was included in each PCR run. To start the reactions, 12.5  $\mu$ L of U-buffer was added to each labeled PCR tube 15 and the tubes were mixed gently by mid-speed vortex before being placed on the PCR machine.

During the PCR thermal cycling procedure, the PCR tubes were subjected to a starting temperature of 95°C for 4 minutes, followed by 5 cycles (95°C for 30 seconds, 67°C for 45 seconds, and 72°C for 1 minute), followed by 20 27 cycles (95°C for 30 seconds, 69°C for 45 seconds, and 72°C for 1 minute), followed by 7 minutes at 72°C. To melt the DNA double strands, each PCR tube was incubated at 96°C for 2 minutes. In addition, the branched DNA formation was initiated by placing the denatured DNA at 65°C for 30 minutes and then incubating the reaction at 4°C for 30 minutes.

25

The false positive samples were analyzed using the Dig-Primer1, Bt-Primer1, Primer4, and Primer5 primers. In each case, the samples were correctly identified as either negative (-/-) or positive (+/-). These results demonstrate that

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the intronic sequences after exon 10 potentially contain polymorphisms since the elimination of intronic sequences avoided the false positive results.

Example 2 - Factor V Leiden Detection from Whole Blood

Stable nucleic acid branching structures were detected in a cell lysate  
5 mixture containing amplified nucleic acid. The presence of stable branching structures indicates that the region amplified corresponds to a region containing heterologous alleles.

To prepare a cell lysate, 200 µL of whole blood (WB) was placed into a 0.5 mL tube and an equal volume (200 µL) of 0.14M NH<sub>4</sub>CL, 0.017M Tris, pH 10 7.4 was added. The tube then was vortexed for 5 seconds and placed on ice for 5 minutes. After removing the tube from the ice, the tube was centrifuged at 1000 x g for 1 minute. When a fixed speed microcentrifuge was used, the tube was pulsed in 3 second intervals four times for a total pulse time of about 12 seconds.

After centrifugation, the supernatant was removed and discarded. The 15 remaining pellet was washed three times as follows: 200 µL of 1X PCR Buffer (2 mM MgCl<sub>2</sub>; 20mM Tris (pH8); 10mM KCl; 0.1% Triton X-100; and 6mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) was added, the tube vortexed briefly and centrifuged (1000 x g for 1 minute, or when a fixed speed microcentrifuge was used, pulsed in 3 second intervals four times for a total pulse time of about 12 seconds), and the supernatant 20 removed and discarded. Twenty (20) µL 1X TE and 20 µL GeneReleaser™ (BioVentures, Inc) were added to the remaining pellet. The tube then was vortexed for 5 seconds and microwaved for seven minutes using a microwave oven (4500 Watt-Minutes total). Following the microwave treatment, the tube was centrifuged for 30 seconds, and 20 µL of the supernatant containing the nucleic acid released 25 from the cells was transferred to a fresh tube. A 2.5 µL aliquot of the supernatant containing the nucleic acid was used per 51 µL PCR reaction.

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For each PCR reaction, the PCR reaction buffer (L-Buffer) and polymerase working solution (U-Buffer) were as follows:

	L-Buffer	U-Buffer
<b>Nuclease-free water</b>	31.6 µL	12.25 µL
<b>10X polymerase buffer</b>	3.75 µL	1.25 µL
<b>Primer mix</b>	4 µL	
<b>10 mM dNTP mix</b>	0.5 µL	
<b>Native <i>Pfu</i> polymerase</b>		0.6 µL
<b>Total volume per reaction</b>	36 µL	12.5 µL

- To setup each PCR reaction, 36 µL of L-Buffer, 2.5 µL of either DNA 10 (around 10 ng/µL) or cell lysate, and 12.5 µL of U-Buffer were combined in a PCR tube that was then placed on the PCR machine. During the PCR thermal cycling procedure, the PCR tubes were subjected to a starting temperature of 95°C for 4 minutes, followed by 5 cycles (95°C for 30 seconds, 60°C for 45 seconds, and 72°C for 1 minute), followed by 30 cycles (95°C for 30 seconds, 65°C for 45 15 seconds, and 72°C for 1 minute), followed by 7 minutes at 72°C. Following nucleic acid amplification, additional MgCl<sub>2</sub> was added to each tube to a final concentration of 4mM. To melt the DNA double strands, each PCR tube was incubated at 96°C for 2 minutes. In addition, the branched DNA formation was initiated by placing the denatured DNA at 65°C for 30 minutes and then incubating 20 the reaction at 4°C for 30 minutes.

The methods used to detect nucleic acid branching structures were similar to those used in Example 1.

Three of three samples containing known mutations were correctly identified (Table III). Two samples were classified as false positive, and 17 of 19 25 samples were correctly classified as wild-type (non-mutation). This method did not identify any false negative samples. Thus, 5 (three true positives and two false positives) of 22 samples tested (about 23%) would require mutational confirmation

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analysis. In a repeat experiment, one of the false positive samples were re-classified as negative. Pico Green Fluorescence, measured in relative fluorescence units (rfu's), was used to verify PCR amplification and thus prevent PCR failures from being mis-identified as wild-type.

5 Table III. Factor V analysis directly from whole blood samples

	Coag Lab Result	LOCI counts	Pico Green rfu's	Loci Results
	+/-	102991	7,768	+
	-/-	7140	5,738	-
	+/-	226741	25,986	+
10	-/-	5126	21,676	-
	-/-	23713	14,558	-
	-/-	2447	11,902	-
	-/-	3251	6,776	-
	-/-	5126	10,810	-
15	-/-	56177	11,656	FP; repeated = neg
	-/-	2727	10,750	-
	-/-	2424	5,738	-
	-/-	1601	14,172	-
	-/-	7651	9,056	-
20	-/-	2749	10,014	-
	-/-	3863	12,894	-

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-/-	3465	11,292	-
-/-	4163	6,720	-
-/-	117,483	10,092	FP
+/-	143385	11,446	+
-/-	7759	6,436	-
-/-	3902	9,964	-

Example 3 - Cystic Fibrosis Exon 10 Mutation Detection from Whole Blood

Stable nucleic acid branching structures were detected in a cell lysate mixture containing amplified nucleic acid. The presence of stable branching structures indicates that the region amplified corresponds to a region containing heterologous alleles. For the detection of cystic fibrosis mutations, the cell lysate was prepared as described in Example 2. In addition, the PCR reactions were performed using 2 µL of cell lysate and 38 µL of a mixture containing 1X polymerase buffer, 0.4mM dNTPs, 0.625mM each primer, 0.0156 units/mL native *Pfu* polymerase, and 0.0156 units/mL *exo Pfu*. The PCR amplification conditions were as follows: starting temperature of 96°C for 2 minutes, followed by 40 cycles (95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 45 seconds), followed by 7 minutes at 72°C. Following nucleic acid amplification, additional MgCl<sub>2</sub> was added to each tube to a final concentration of 4mM.

The oligonucleotide primers amplified a 200 base pair region of Exon 10 of the cystic fibrosis gene. The two forward primers (Dig-Primer12 and Bt-Primer12) were designed to have the same sequence (5'-CTCAGTTTCCTGGATTATGCC-3'; SEQ ID NO:12) with a different binding agent attached at the 5'-end: one having digoxigenin (Dig-Primer12) and the other having biotin (Bt-Primer12).

The two lower primers (Primer13 and Primer14) also target the cystic fibrosis gene. These two primers were designed to have an identical sequence at

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the 3'-end and a different sequence at the 5'-end of the primer. The sequence of Primer13 (SEQ ID NO:13) and Primer14 (SEQ ID NO:14) is as follows with the identical sequences being underlined:

- 5'-ACCATGCTCGAGATTACGAGCTAACCGATTGAATATGGAGCC-3' (SEQ  
5 ID NO:13)
- 5'-GATCCTAGGCCTCACGTATTCTAACCGATTGAATATGGAGCC-3' (SEQ  
ID NO:14)

After completion of PCR, branch migration was carried out under these conditions: denaturation at 96°C for 2 minute and branch migration at 65°C for 30 minutes followed by a 4°C hold. Upon completion of branch migration, 3 µL of amplified product was mixed with 100 µL of 1X LOCI buffer (10mM Tris-HCl, 50mM KCl, 4mM MgCl<sub>2</sub>, 200µg/mL BSA) containing of chemiluminescent sensitizer beads (0.25mg/mL) and acceptor beads (0.25mg/mL). These reactions were incubated in the dark at 37°C for 30 minutes. The samples were illuminated, 15 and chemiluminescence was detected using the DNA LOCI reader.

Two of two samples containing known mutations were correctly identified (Table IV). One sample was classified as a false positive, and 19 of 20 samples were classified correctly as wild-type including two heterozygous mutations in exon 4. This method did not identify any false negative samples.  
20 Thus, three (two true positives and one false positive) of 22 samples tested (about 14%) would require mutational confirmation analysis.

Table IV. Cystic Fibrosis gene exon 10 molecular genetics using whole blood samples

Molecular Genetics Results	LOCI counts	Pico Green (rfu's)	LOCI Results Exon 10
neg	2263	12,296	-
neg	2613	9,466	-

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	neg	2330	8,196	-
	neg	1990	8,054	-
	neg	2015	8,968	-
	Hetero (Exon 4)	2110	8,004	-
5	Hetero (Exon 10)	230041	9,258	+
	neg	2169	9,304	-
	neg	2856	13,702	-
	neg	2650	11,624	-
	neg	2082	7,748	-
10	Hetero (Exon 4)	2619	10,204	- (Exon 10)
	neg	2772	9,898	-
	neg	2847	7,420	-
	neg	3259	8,636	-
	neg	1986	5,698	-
15	neg	2525	9,348	-
	neg	2585	10,304	-
	neg	119352	7,342	FP - confirmed to be G1716A polymorphism
	neg	2053	8,114	-
	Hetero (Exon 10)	241577	10,364	+
20	neg	2371	10,972	-

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Example 4 - Factor V Leiden and Cystic Fibrosis Exon 10 Mutation

Detection using a Sandwich Immunoassay

- To detect Factor V Leiden mutations using a microtiter plate format, Dig-Primer1, Bt-Primer1, Primer6, and Primer7 primers were used to amplify a 258 base pair fragment containing the Factor V Leiden site. Primer6 and Primer7 were designed to have an identical sequence at the 3'-end and a different sequence at the 5'-end of the primer. The sequence of Primer6 (SEQ ID NO:6) and Primer7 (SEQ ID NO:7) is as follows with the identical sequences being underlined:
- 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125 130 135 140 145 150 155 160 165 170 175 180 185 190 195 200 205 210 215 220 225 230 235 240 245 250 255 260 265 270 275 280 285 290 295 300 305 310 315 320 325 330 335 340 345 350 355 360 365 370 375 380 385 390 395 400 405 410 415 420 425 430 435 440 445 450 455 460 465 470 475 480 485 490 495 500 505 510 515 520 525 530 535 540 545 550 555 560 565 570 575 580 585 590 595 600 605 610 615 620 625 630 635 640 645 650 655 660 665 670 675 680 685 690 695 700 705 710 715 720 725 730 735 740 745 750 755 760 765 770 775 780 785 790 795 800 805 810 815 820 825 830 835 840 845 850 855 860 865 870 875 880 885 890 895 900 905 910 915 920 925 930 935 940 945 950 955 960 965 970 975 980 985 990 995 1000 1005 1010 1015 1020 1025 1030 1035 1040 1045 1050 1055 1060 1065 1070 1075 1080 1085 1090 1095 1100 1105 1110 1115 1120 1125 1130 1135 1140 1145 1150 1155 1160 1165 1170 1175 1180 1185 1190 1195 1200 1205 1210 1215 1220 1225 1230 1235 1240 1245 1250 1255 1260 1265 1270 1275 1280 1285 1290 1295 1300 1305 1310 1315 1320 1325 1330 1335 1340 1345 1350 1355 1360 1365 1370 1375 1380 1385 1390 1395 1400 1405 1410 1415 1420 1425 1430 1435 1440 1445 1450 1455 1460 1465 1470 1475 1480 1485 1490 1495 1500 1505 1510 1515 1520 1525 1530 1535 1540 1545 1550 1555 1560 1565 1570 1575 1580 1585 1590 1595 1600 1605 1610 1615 1620 1625 1630 1635 1640 1645 1650 1655 1660 1665 1670 1675 1680 1685 1690 1695 1700 1705 1710 1715 1720 1725 1730 1735 1740 1745 1750 1755 1760 1765 1770 1775 1780 1785 1790 1795 1800 1805 1810 1815 1820 1825 1830 1835 1840 1845 1850 1855 1860 1865 1870 1875 1880 1885 1890 1895 1900 1905 1910 1915 1920 1925 1930 1935 1940 1945 1950 1955 1960 1965 1970 1975 1980 1985 1990 1995 2000 2005 2010 2015 2020 2025 2030 2035 2040 2045 2050 2055 2060 2065 2070 2075 2080 2085 2090 2095 2100 2105 2110 2115 2120 2125 2130 2135 2140 2145 2150 2155 2160 2165 2170 2175 2180 2185 2190 2195 2200 2205 2210 2215 2220 2225 2230 2235 2240 2245 2250 2255 2260 2265 2270 2275 2280 2285 2290 2295 2300 2305 2310 2315 2320 2325 2330 2335 2340 2345 2350 2355 2360 2365 2370 2375 2380 2385 2390 2395 2400 2405 2410 2415 2420 2425 2430 2435 2440 2445 2450 2455 2460 2465 2470 2475 2480 2485 2490 2495 2500 2505 2510 2515 2520 2525 2530 2535 2540 2545 2550 2555 2560 2565 2570 2575 2580 2585 2590 2595 2600 2605 2610 2615 2620 2625 2630 2635 2640 2645 2650 2655 2660 2665 2670 2675 2680 2685 2690 2695 2700 2705 2710 2715 2720 2725 2730 2735 2740 2745 2750 2755 2760 2765 2770 2775 2780 2785 2790 2795 2800 2805 2810 2815 2820 2825 2830 2835 2840 2845 2850 2855 2860 2865 2870 2875 2880 2885 2890 2895 2900 2905 2910 2915 2920 2925 2930 2935 2940 2945 2950 2955 2960 2965 2970 2975 2980 2985 2990 2995 3000 3005 3010 3015 3020 3025 3030 3035 3040 3045 3050 3055 3060 3065 3070 3075 3080 3085 3090 3095 3100 3105 3110 3115 3120 3125 3130 3135 3140 3145 3150 3155 3160 3165 3170 3175 3180 3185 3190 3195 3200 3205 3210 3215 3220 3225 3230 3235 3240 3245 3250 3255 3260 3265 3270 3275 3280 3285 3290 3295 3300 3305 3310 3315 3320 3325 3330 3335 3340 3345 3350 3355 3360 3365 3370 3375 3380 3385 3390 3395 3400 3405 3410 3415 3420 3425 3430 3435 3440 3445 3450 3455 3460 3465 3470 3475 3480 3485 3490 3495 3500 3505 3510 3515 3520 3525 3530 3535 3540 3545 3550 3555 3560 3565 3570 3575 3580 3585 3590 3595 3600 3605 3610 3615 3620 3625 3630 3635 3640 3645 3650 3655 3660 3665 3670 3675 3680 3685 3690 3695 3700 3705 3710 3715 3720 3725 3730 3735 3740 3745 3750 3755 3760 3765 3770 3775 3780 3785 3790 3795 3800 3805 3810 3815 3820 3825 3830 3835 3840 3845 3850 3855 3860 3865 3870 3875 3880 3885 3890 3895 3900 3905 3910 3915 3920 3925 3930 3935 3940 3945 3950 3955 3960 3965 3970 3975 3980 3985 3990 3995 4000 4005 4010 4015 4020 4025 4030 4035 4040 4045 4050 4055 4060 4065 4070 4075 4080 4085 4090 4095 4100 4105 4110 4115 4120 4125 4130 4135 4140 4145 4150 4155 4160 4165 4170 4175 4180 4185 4190 4195 4200 4205 4210 4215 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- To setup each PCR reaction, 35 µL of L-Buffer, 2.5 µL of either DNA (around 10 ng/µL) or cell lysate, and 12.5 µL of U-Buffer were combined in a PCR tube that was then placed on the PCR machine. The PCR amplification conditions were as follows: 95°C for 2 minutes, followed by 40 cycles (95°C for 15 seconds, 64°C for 30 seconds, and 72°C for 1 minute), followed by a final extension for 7 minutes at 72°C. After the PCR amplifications, additional MgCl<sub>2</sub> was added to a final concentration of 4 mM. To melt the DNA double strands,

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each PCR tube was incubated at 95°C for 2 minutes. In addition, the branched DNA formation was initiated by placing the denatured DNA at 65°C for 30 minutes and then the reaction was maintained at 4°C until analysis.

The microtiter plate detection format was performed as follows. The 5 amplification product from each PCR reaction was diluted 1:100 in 1X PBS, 0.1% gelatin. A 100 µL aliquot of each diluted sample then was added to a well of a streptavidin-coated plate (Boehringer Mannheim). After sealing, the plate was incubated in a 37°C shaking incubator for one hour. After incubation, each well was washed four times with 1X PBS containing 0.05% Tween-20. Once washed, 10 100 µL of anti-Dig antibody conjugated with POD (horse radish peroxidase; antibody concentration of 16.67 milli units/mL in 1X PBS containing 0.05% Tween-20; Boehringer Mannheim) was added to each well, and the plate sealed and incubated in a 37°C shaking incubator for 30 minutes. After incubation, each well was washed four times with 1X PBS containing 0.05% Tween-20, and 100 µL of 15 diluted substrate was added to each well. The results then were read from the plate on a luminometer (Microlumat LB 96 P; EG&G Berthold).

In this analysis, 129 samples were analyzed. Based on a bi-PASA analysis, 78 samples were heterozygous for Factor V Leiden, 16 samples were homozygous for Factor V Leiden, and 35 samples were homozygous for wild-type 20 Factor V. Using the Dig-Primer1, Bt-Primer1, Primer6, and Primer7 primers to amplify Factor V nucleic acid from whole blood and a sandwich immunoassay to detect stable branching structures, 79 samples were identified as positive for stable branching structures, 16 samples were identified as homozygous (absence of stable branching structures converted to presence of branching structures upon mixing 25 with known homologous wild-type amplification product), and 34 samples were identified as homozygous for wild-type Factor V. The one false positive sample that was classified as homozygous for wild-type Factor V based on bi-PASA was in fact heterozygous for a mutation other than the Leiden mutation. Specifically, this sample was found to contain a G to A mutation on one allele at position 1628 30 based on sequencing data.

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#### OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by 5 the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

**WHAT IS CLAIMED IS:**

1. A composition comprising oligonucleotides, wherein said composition amplifies a region of Factor V genomic sequence during a nucleic acid amplification reaction such that amplified products that are heterologous form stable branching structures, and wherein said region contains a potential mutation site.
2. The composition of claim 1, wherein said composition comprises two oligonucleotides, wherein said two oligonucleotides are identical in nucleic acid sequence, each of said two oligonucleotides having a different label attached at the 10 5' end.
3. The composition of claim 2, wherein said label comprises a binding agent selected from the group consisting of biotin and digoxigenin.
4. The composition of claim 2, wherein each of said two oligonucleotides has a nucleic acid sequence as set forth in SEQ ID NO:1.
- 15 5. The composition of claim 1, wherein said composition comprises two oligonucleotides each having a 5' portion and a 3' portion, wherein said 5' portions of said two oligonucleotides are different in nucleic acid sequence, and said 3' portions of said two oligonucleotides are identical in nucleic acid sequence, and wherein said 3' portions hybridize to a sequence flanking said mutation site during 20 said nucleic acid amplification reaction.
6. The composition of claim 5, wherein one of said two oligonucleotides has a nucleic acid sequence as set forth in SEQ ID NO:2, 3, 4, 5, 6, or 7.

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7. The composition of claim 1, wherein said amplified products are less than about 300 bases in length.
8. The composition of claim 7, wherein one of said oligonucleotides has a nucleic acid sequence as set forth in SEQ ID NO:4, 5, 6, or 7.
- 5 9. The composition of claim 1, wherein said oligonucleotides produce false positive results in a LOCI mutation detection assay less frequently than when Dig-Primer1, Bt-Primer1, Primer2, and Primer3 primers are used in said LOCI mutation detection assay.
10. The composition of claim 9, wherein said oligonucleotides comprise Dig-Primer1, Bt-Primer1, Primer4, and Primer5.
11. An oligonucleotide, wherein said oligonucleotide consists of the sequence as set forth in SEQ ID NO:1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14.
12. A method of detecting heterologous Factor V alleles in an organism, said method comprising:
  - 15 (a) amplifying a region of Factor V nucleic acid from said organism using oligonucleotides such that amplified products that are heterologous form stable branching structures, wherein said region contains a potential mutation site; and
  - (b) detecting the presence or absence of said stable branching structures,
- 20 wherein the presence of said stable branching structures is indicative of the presence of said heterologous Factor V alleles in said organism.
13. The method of claim 12, wherein LOCI is used for said detecting step.

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14. The method of claim 12, wherein a sandwich immunoassay is used for said detecting step.

15. A method of detecting homozygous mutant Factor V alleles in an organism, said method comprising:

5 (a) amplifying a region of Factor V nucleic acid from said organism using oligonucleotides to form amplification products, wherein said region contains a potential mutation site;

10 (b) contacting said amplification products with a solution to form a mixture, wherein said solution comprises homozygous wild-type Factor V products amplified using said oligonucleotides; and

15 (c) detecting the presence or absence of stable branching structures in said mixture, said stable branching structures being formed between said amplification products and said homozygous wild-type Factor V products, the presence of said stable branching structures being indicative of the presence of said homozygous mutant Factor V alleles in said organism.

16. The method of claim 15, wherein LOCI is used for said detecting step.

17. The method of claim 15, wherein a sandwich immunoassay is used for said detecting step.

18. A diagnostic kit for nucleic acid extraction-free detection of  
20 heterologous alleles in an organism, said kit comprising a cell lysis reagent and two oligonucleotides, wherein said two oligonucleotides are identical in nucleic acid sequence, each of said two oligonucleotides having a different label attached at the 5' end, wherein said two oligonucleotides hybridize to a first region of genomic sequence during a nucleic acid amplification reaction, and wherein said first region  
25 flanks a potential mutation site.

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19. The kit of claim 18, wherein said organism is a human.
20. The kit of claim 18, wherein said cell lysis reagent comprises an ingredient that inactivates a polymerase inhibitor.
21. The kit of claim 18, wherein said two oligonucleotides have a sequence  
5 as set forth in SEQ ID NO:1.
22. The kit of claim 18, wherein said label comprises a binding agent selected from the group consisting of biotin and digoxigenin.
23. The kit of claim 18, wherein said genomic sequence comprises a Factor V genomic sequence.
- 10 24. The kit of claim 18, wherein said genomic sequence comprises a cystic fibrosis genomic sequence.
25. The kit of claim 18, wherein said kit comprises two additional oligonucleotides each having a 3' portion and a 5' portion, said 5' portions being different in nucleic acid sequence, and said 3' portions being identical in nucleic  
15 acid sequence, wherein said 3' portions hybridize to a second region of said genomic sequence during said nucleic acid amplification reaction, said second region being located on a side of said potential mutation site that is opposite the side of said first region.
26. The kit of claim 25, wherein said second region is downstream of said  
20 potential mutation site.
27. The kit of claim 25, wherein said second region is upstream of said potential mutation site.

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28. The kit of claim 25, wherein one of said two additional oligonucleotides has a sequence as set forth in SEQ ID NO:2, 3, 4, 5, 6, or 7.

29. A method of comparing nucleic acid sequences of alleles in an organism, said method comprising:

5 (a) amplifying a nucleic acid region of said alleles in a cell lysate to form a cell lysate mixture having amplification products;

(b) analyzing said cell lysate mixture for the presence or absence of stable nucleic acid branching structures; and

10 (c) classifying said alleles as heterologous if said stable nucleic acid branching structures are present in said cell lysate mixture.

30. A method of detecting homozygous mutant alleles in an organism, said method comprising:

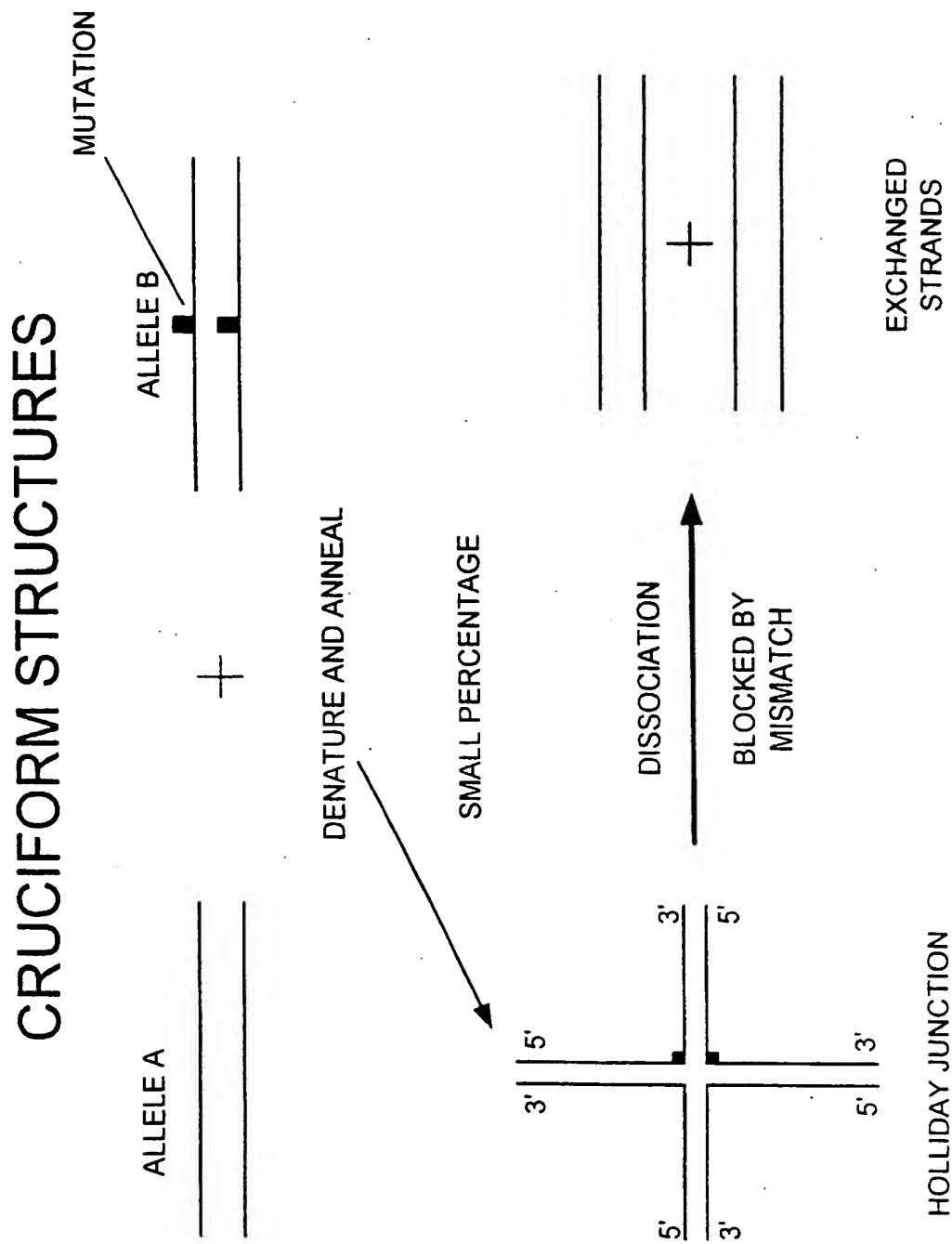
(a) amplifying a nucleic acid region of said alleles in a cell lysate using oligonucleotides to form a cell lysate mixture having amplification products;

15 (b) contacting said cell lysate mixture with a solution to form a second mixture, wherein said solution comprises homozygous wild-type products amplified from homozygous wild-type alleles using said oligonucleotides;

(c) analyzing said second mixture for the presence or absence of stable nucleic acid branching structures; and

20 (d) classifying said alleles as homozygous mutant alleles if said stable nucleic acid branching structures are present in said second mixture.

FIG. 1



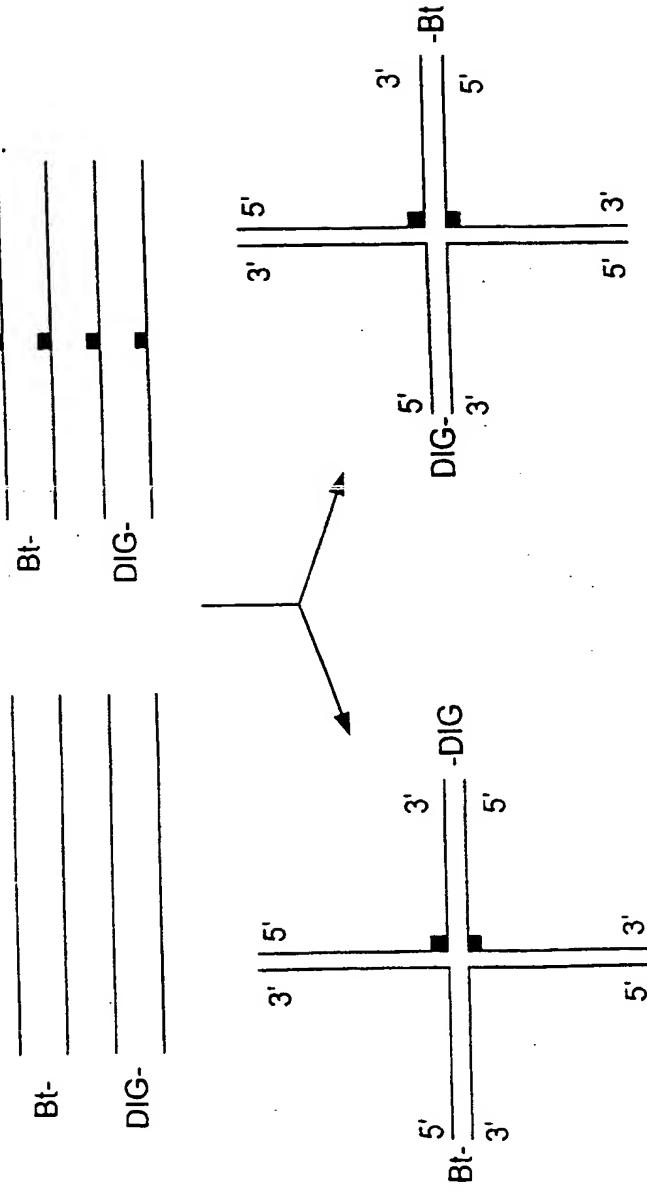
SUBSTITUTE SHEET (RULE 26)

# CRUCIFORM STRUCTURE TAGGING

**FIG. 2**

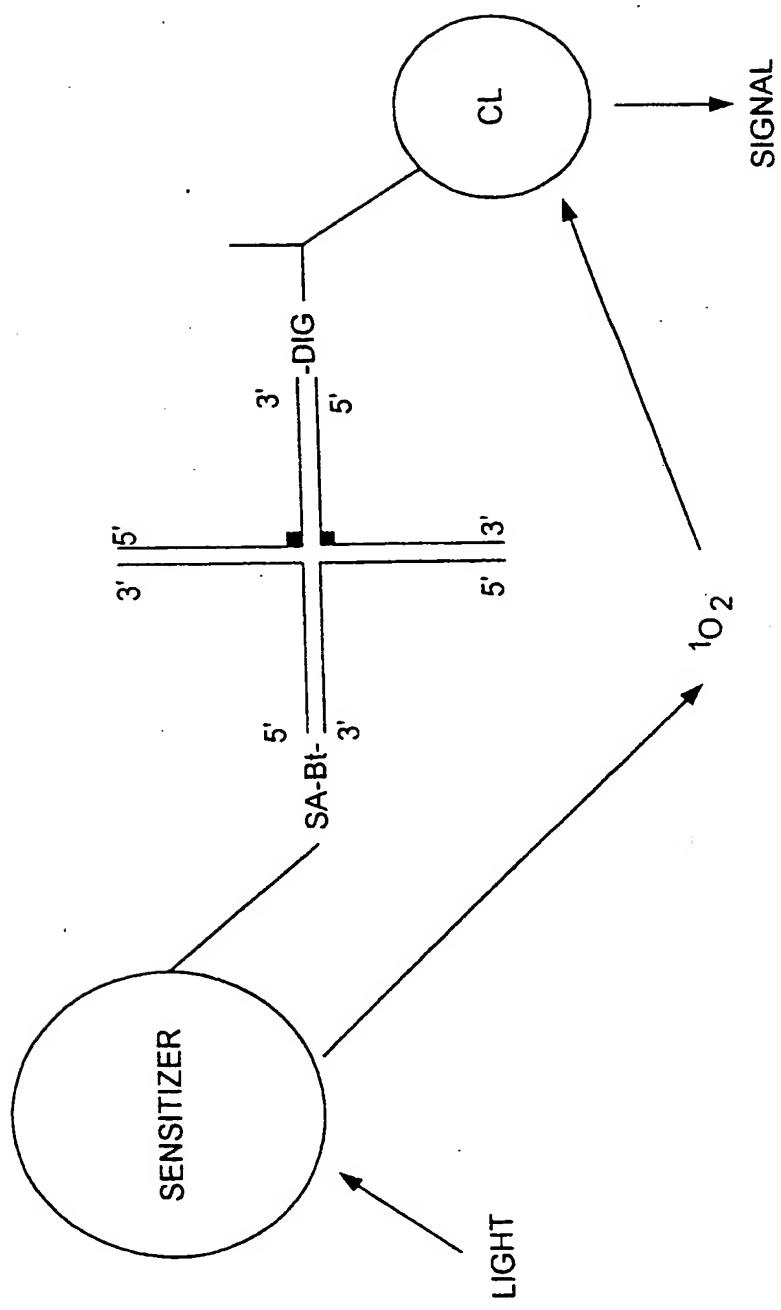


**AMPLIFICATION WITH 5'-Bt-PRIMER + 5' DIG-PRIMER**



**FIG. 3**

**CRUCIFORM STRUCTURE DETECTION**



**SUBSTITUTE SHEET (RULE 26)**

## SEQUENCE LISTING

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RESEARCH

<120> SIMPLIFIED MUTATION DETECTION

<130> 07039/0138WO1

<150> 60/071,965

<151> 1998-01-20

<150> 60/082,024

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/01030

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C07H 21/02, 21/04  
US CL :435 6,91.1; 530/23.1, 24.33

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435 6,91.1; 530/23.1, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JENNY, R.J. et al. Complete cDNA and derived amino acid sequence of human factor V. PNAS. July 1987, Vol. 84, pages 4846-4850, see entire document.	1-30
Y	KANE, W.H. et al. Cloning of cDNAs coding for the Heavy Chain Region and Connecting Region of Human Factor V, a Blood Coagulation Factor with Four types of Internal Repeats. Biochemistry. 1987, Vol. 26, pages 6508-6514, see entire document.	1-30
Y	CRIPPE, L.D. et al. Structure of the Gene for Human Coagulation Factor V. Biochemistry. 1992, Vol. 31, No. 15, pages 3777-3785, see entire document.	1-30

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	*T*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

27 FEBRUARY 1999

Date of mailing of the international search report

25 MAR 1999

Name and mailing address of the ISA/US  
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Box PCT  
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